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The importance of p53 on prostate cancer is underlined by clinical observations that p53 alteration can be seen in most metastatic prostate cancers. The finding that re-introduction of wild-type p53 can cause growth arrest of prostate cancer cells further support the role of p53 in prostate tumor suppression. Therefore, to study on prostate tumor development and a therapeutic strategy targeting p53, it is necessary to understand how p53 is activated. To study this, we focus the p53 acetylation, which has been found as a potential mechanism of p53 activation, and investigate how acetylation controls the activity of p53. In this report, we provide the evidence that acetylation regulates p53 subcellular localization. Our study identifies acetylation as a novel mechanism that regulates p53 nucleus-cytoplasm trafficking by neutralizing C-terminal lysine residues, which in turn, controls the oligomerization-dependent nuclear export machinery. Although further investigations are needed to address the significance of acetylation-induced p53 trafficking in prostate cancer, our findings provide the basis for a more powerful therapy for prostate tumor suppression.

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Introduction

Impairment of the activity of tumor suppressor p53 plays a critical role in most prostate cancers. In therapy, one critical problem is that most prostate cancers eventually become resistance to the standard androgen ablation hormonal therapy. Interestingly, it has been shown that androgen ablation-induced apoptosis in prostate gland is greatly diminished in the p53-deficient mouse (1). The role of p53 in prostate tumor development is further supported by finding that re-introduction of wild type p53 can cause growth arrest of prostate cancers cell lines (2). These results provide not only a potential molecular mechanism of resistance to hormone ablation therapy in advanced prostate tumors, which often harbor p53 mutations (3), but also a strategy of therapeutic agents that can enhance p53 activity for prostate tumor suppression. To study this, it is necessary to understand how p53 is activated. Recent finding show that p53 acetylation might play a role in p53 activation (reviewed in (4) (5)). Importantly, the signal transduction pathway leading to p53 actylation is functional in prostate cells, suggesting that this modification could be important for prostate tumor suppression. In this research, we will address to decipher the functional significance of p53 acetylation (**Aim1**). It has been shown that subcellular localization of p53 is important for p53 activation. Therefore, we will also address to characterize the role and significance of acetylation in regulating p53 subcellular localization (**Aim2**). Our research will provide important information on drag discovery targeting p53 activity by manipulating its acetylation state for prostate tumor suppression.

Body

Aim 1. To decipher the functional significance of p53 acetylation.

To address this, we are now trying to determine the role of p53 acetylation in p53 mediated transcriptional regulation, growth arrest and apoptosis (**Aim1 I**). We are assessing the phenotype of an acetylation-deficent p53 6KR mutant by examining G1 arrest, apoptosis and transcriptional activity for target genes in prostate cancer cells. If we finish this, we will move to next aim in which we will determine if p53 acetylation regulates its association with target gene promoters in vivo (**Aim1 II**) and whether acetylation modulates p53 protein-protein interaction (**Aim1 III**).

Aim 2.To characterize the role and significance of acetylation in regulating p53 subcellular localization.

Although the importance of p53 intracellular trafficking in the regulation of p53 activity has been accumulating (6) (7) (8), little is known about how p53 intracellular trafficking is regulated. Surprisingly, we found that p300-mediated acetylation promotes cytoplasmic accumulation of p53. First, we have performed to decipher how acetylation regulates the cytoplasmic accumulation of p53 (**Aim2 III**). Here we report evidence that lysine acetylation can control p53 subcellular trafficking and p53 oligomerization. Our findings also suggest a potential novel threshold mechanism wherein the acetylation level of p53 serves as a signal that promotes p53 export to the cytoplasm.

C-terminal lysine residues are required for efficient acetylation-induced cytoplasmic accumulation of p53

So far, we have already demonstrated that p300-mediated p53 acetylation promotes p53 nuclear export. The significant accumulation of endogenous p53 in the cytoplasm in response to UV-irradiation or Hypoxia when deacetylation of p53 is suppressed supports our conclusion. Importantly, MDM2, a negative regulator of p53, has been known to stimulate p53 nuclear export by promoting p53 ubiquitination (9) (10). As p300 functionally interacts with MDM2 (11), we asked if p300-induced cytoplasmic accumulation of p53 requires MDM2 by examining subcellular localization of p53 with co-expressing p300 in MDM2-deficient MEF cells. As shown in Figure 1, in the absence of MDM2, p300 is still capable of stimulating the cytoplasmic accumulation of p53. This result indicates that p300-mediated cytoplasmic accumulation of p53 is independent of MDM2, and supports the idea that p300 affects subcellular localization of p53 in an acetylation-dependent manner, likely by directly acetylating p53. To further investigate this possibility, we determined whether lysine (K) residues known to be acetylated by p300 are required for acetylation-induced cytoplasmic accumulation of p53. We, therefore, evaluated the subcellular distribution of p53 KR mutants in response to p300. As shown in Figure 2B, when expressed alone, the localization of these p53 KR mutants is almost entirely nuclear and indistinguishable from wild type p53. However, in response to ectopically expressed p300, the number of cells that show a cytoplasmic accumulation of the 5KR (51%) and 6KR (30%) p53 mutants is markedly reduced compared to that of wild type p53 (84%). These data indicate that the lysine residues acetylated by p300 are required to mediate a maximal p53 nuclear exit in response to p300.

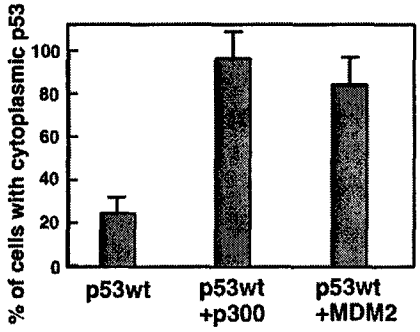

Figure 1. Acetylation-mediated p53 nuclear export is not MDM2-dependent. The percent of cells with cytoplasmic p53 was determined by counting 200 cells from each transfection. Note that cytoplasmic accumulation of p53 is observed in MDM2(-/-) cells co-transfected with p300.

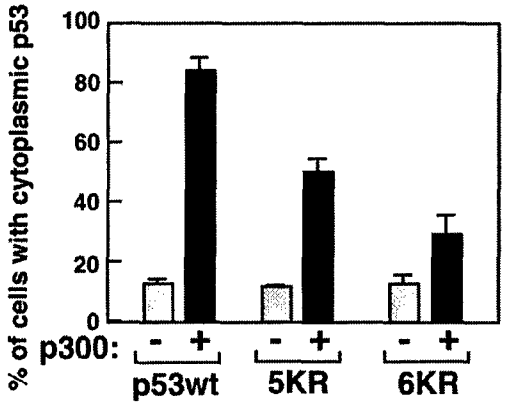

Figure 2. The C-terminal lysines of p53 are involved in the acetylation-mediated nuclear export of p53. The loss of available acetylation sites results in a reduction in the percentage of cells with cytoplasmic p53. Wild-type p53 or the KR mutants were transfected into H1299 cells alone or co-transfected with p300 as indicated. The percent of cells with cytoplasmic p53 was determined as in Figure 1. Note that a cytoplasmic accumulation of the 6KR p53 mutants is markedly reduced in response to co-expressed p300.

Neutralization of positively charged lysine residues in the C-terminus regulates subcellular localization of p53

Our data suggests that, similar to ubiquitination, the acetylation of p53 also leads to its nuclear exit. Although, ubiquitin and acetyl groups have little in common structurally, they both modify the ε amino group of the lysine residue and neutralize its charge. We therefore hypothesize that acetylation promotes p53 nuclear exit by neutralizing the charge of the targeted lysine residues. To test this hypothesis, we generated charge-neutralizing mutations by converting lysine (K) residues known to be acetylated and ubiquitinated to alanine (A), either individually or in combination, and evaluated their subcellular localization. As shown in Figure 3, the subcellular localization of the 2KA and several 3KA (3KA-1, 3KA-2 and 3KA-3) mutants is similar to that of wild type p53 and is mostly nuclear (Figure 3B and C). In contrast, when four lysine residues are mutated in three different combinations (4KA-1, 4KA-2 and 4KA-3), these p53 mutants clearly began to accumulate in the cytoplasm. The conversion of five lysine residues (5KA) lead to a further increase in the number of cells with cytoplasmic p53 staining (Figure 3B and C). Furthermore, the intensity of the wild type p53 protein detected in the cytoplasm was much weaker than that of the 4KA and 5KA mutants (data not shown). Importantly, the population of cells showing cytoplasmic p53 4KA and 5KA mutants can be effectively eliminated upon treatment with LMB (Figure 3B and C), supporting the idea that nuclear export is required for cytoplasmic accumulation of the 4KA and 5KA p53 mutants. Together, these observations indicate that the degree of p53 cytoplasmic accumulation is proportional to the number of lysine residues neutralized.

Therefore, these results suggest that acetylations modulate p53 subcellular localization by modifying the positive charge of specific lysine residues at the C-terminus of p53.

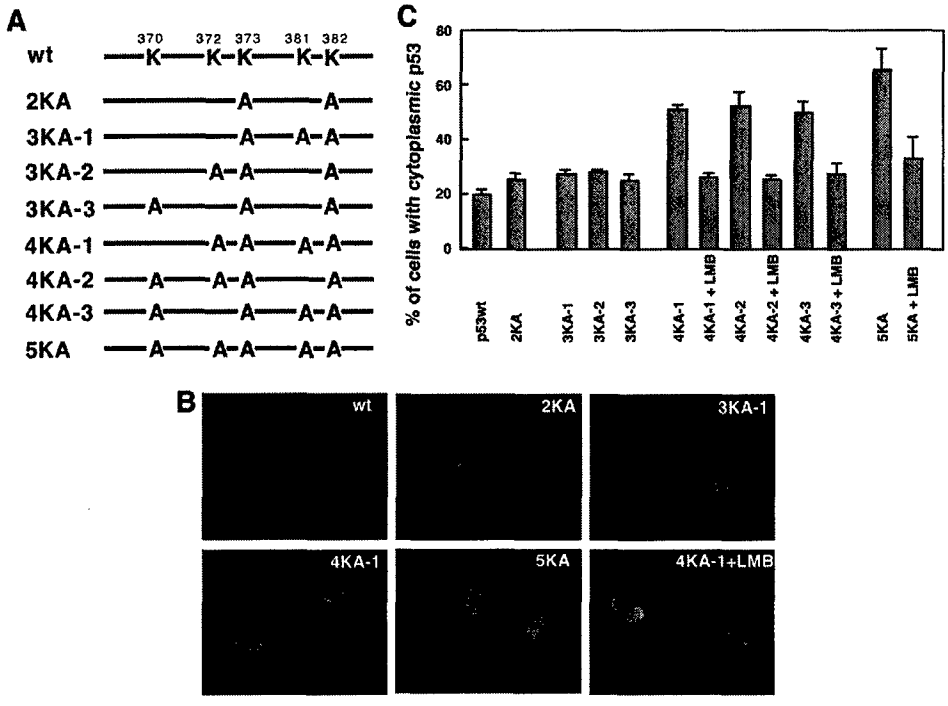

Figure 3. Neutralization of positively charged lysine residues in the C-terminus of p53 regulates subcellular localization of p53. **(A)** Schematic representation of the p53 lysine (K) to alanine (A) mutants. **(B)** Subcellular localization of wild-type p53 and KA mutants transfected into H1299 cells. LMB treatment was performed as described in Figure 1A. **(C)** An increase in the number of KA mutations results in an increase in the percent of cells with cytoplasmic p53. Results were determined as in Figure 1B. Note that in contrast to 3KA mutants, cytoplasmic accumulation of 4KA mutants markedly increases.

Interestingly, although the conversion of three different combinations of four lysines known to be targets of acetylation and ubiquitination to alanines (4KA mutants) promotes cytoplasmic accumulation of p53, the conversion of only three lysines with different combinations (3KA) has little effect (Figure 3B and C). This result suggests that there might be a threshold for the activation of p53 export, which is determined by the overall charge provided by the lysine cluster at the C-terminus. Thus, the extent of acetylation could decide the subcellular distribution of the activated p53. We suspect that, upon stresses, p53 would be stabilized in the nucleus when less than three lysine residues are acetylated; however when more than four lysine residues are acetylated, presumably in response to prolonged or intense insults, p53 becomes hyperacetylated and exported to the cytoplasm. Further studies are necessary to address the significance of exported p53 in the cytoplasm.

C-terminal lysine charge determines the oligomerization status of p53

The regulation of p53 subcellular localization is believed to be controlled at the levels of its oligomerization status. It was proposed that tetramerization of p53 prevents

both its nuclear import and export (reviewed in (6)). In the case of p53 nuclear export, as the dominant C-terminal NES is located in the oligomerization domain, it was previously suggested that the accessibility of the p53 NES is dependent on its oligomerization status (12). This conclusion is supported by the solution and the crystal structure of the oligomerization domain, which demonstrates that the C-terminal NES is exposed in monomeric or dimeric conformations but it is buried in the p53 tetramers (13) (14) (15). The inter-conversion between the tetrameric and monomeric or dimeric states would therefore determine the availability of the NES and, consequently, the efficiency of p53 export. However, the biochemical basis that controls p53 oligomerization and its regulation were not known. It is likely that the charge of specific lysine residues targeted by acetylation and ubiquitination machinery may be a key determinant of p53 oligomerization. We, therefore, assessed whether modification of the lysine charge activates p53 export by regulating p53 oligomerization status. Based on the observation that the conversion of at least four but not three lysines to alanines promotes p53 cytoplasmic accumulation, recombinant wild type, 3KA, and 4KA (4KA 1-3, Figure 4A) mutant polypeptides encompassing the entire p53 tetramerization domain and lysine rich C-terminus (amino acids 326-393) were tested for their ability to oligomerize. A mutant p53 with four lysines converted to arginines (4KR), which would prevent acetylation but preserve the charge of the lysine, was used as an additional control. As shown in Figure 4, the wild type and 3KA polypeptides dimerized and tetramerized readily (Lanes 2 and 4). In contrast, the 4KA-1, 4KA-2 and 4KA-3 polypeptides completely failed to do so (Lane 6, 8 and 10). This result correlates well with the respective subcellular localization of these mutants and further supports the proposed model that the p53 NES is not accessible in the tetrameric configuration but exposed in the momomeric or dimeric form (12), and that the possibility of a threshold mechanism for the activation of p53 export. Importantly, polypeptides from the 4KR mutant, which is a nuclear protein (data not shown), showed a wild type capacity to oligomerize (Lane 12). Together, these results demonstrate that the oligomerization status of p53 can be controlled by the charge conferred by a defined number of lysine residues and is correlated with p53 subcellular localization. Consistent with our hypothesis that the charge presented by lysine is the critical determinant for p53 export activity, earlier studies also found that the conversion of lysine residues to isoleucine led to the cytoplasmic buildup of p53 (16). Importantly, cytoplasmic accumulation of p53 caused by neutralizing lysine charge patch can be reversed by LMB treatment, further suggesting a dominant role of p53 nuclear export in response to the charge modification (Figure 3 and 4, (17)). We note that, however, it remains possible that charge neutralization of lysine might affect p53 nuclear import as well. Further studies need to address this possibility. Regardless of which mechanism plays a more dominant role, our results support the idea that a charge modification of the lysine residues may dictate the subcellular localization of p53.

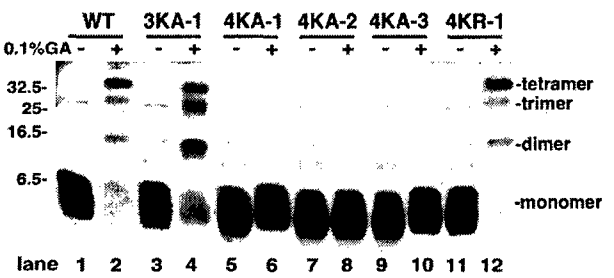

Figure 4. Neutralization of positively charged lysine residues in the C-terminus determines the oligomerization status of p53. Wild-type (lane 1, 2), 3KA (lane 3, 4), 4KA (lane 5, 6) or 4KR (lane 7, 8)

mutant p53 polypeptides consisting of the tetramerization domain (amino acid 326-393) were expressed in *E.coli* as GST fusions. Ten micrograms of GST-cleaved protein were incubated at 37 °C with or without 0.1% glutaraldehyde for 15 min, then analyzed by a 20% SDS-PAGE to separate the p53 monomer from the oligomers. Note that in contrast to 4KR mutants that can oligomerize, 4KA mutants fail to oligomerize.

The biological significance of acetylation-mediated p53 nuclear exit

What is the biological significance of acetylation-mediated p53 nuclear exit? All evidences so far indicate that acetylation positively regulates p53 function (5). Thus, it is logical to speculate that hyperacetylated p53 was delivered to the cytoplasmic compartment to function. Indeed, although p53 is thought to achieve its tumor suppressor activity as a nuclear transcription factor, recent evidence indicates that p53 can also promote apoptosis via a transcriptionally independent mechanism by directly interacting with apoptosis machinery in the cytoplasmic compartment (8). This surprising finding suggests the existence of a mechanism that would transport active p53 to the cytoplasm. One interesting possibility is that acetylation-mediated nuclear export might be involved in this process. In this model, acetylation may be used as a gauge to measure the extent of cellular damage such that hyperacetylation serves as a signal for exporting active p53 to cytoplasm for specific function, such as activating apoptotic program. In this scenario, hyperacetylation of p53 may ensure apoptosis by promoting both transcriptional activation of pro-apoptotic target genes in nucleus and direct binding to BAX in the cytoplasm. To address this, we will determine if cytoplasmic p53 induced by acetylation can induce an association with mitochondria by co-immunostainig and biochemical fractionation (**Aim2 I**). We will also perform to determine if cytoplasmically localized acetylated p53 in important for p53-induced apoptosis by examining the apoptotic activity of 6KR and 5KA mutants (**Aim2 II**). This series of experiments would provide important insight into the function of acetylated p53 and the basis for a more powerful therapy for prostate tumor suppression.

Key Research Accomplishments

Acetylation regulates subcellular localization of p53

C-terminal lysine residues are required for acetylation-induced cytoplasmic accumulation of p53

Neutralization of positively charged lysine residues in C-terminus regulates subcellular localization of p53

C-terminal lysine charge determines the oligomerization status of p53

Reportable Outcomes

None

Conclusions

The understanding of p53 activation provides important information on the strategy for treatment for prostate cancer. We present the evidence that acetylation regulates p53 subcellular localization. Although the importance of acetylation-mediated p53 nuclear export in prostate tumor suppression still awaits further investigation, our study identifies acetylation as a potential novel mechanism that regulates p53 activity by control p53 intracellular trafficking.

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